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ORIGINAL CONTRIBUTION

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Direct protective effects of poly-unsaturated fatty acids, DHA and EPA, against activation of cardiac late sodium current

A mechanism for ischemia selectivity

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Abstract Polyunsaturated fatty acids (PUFAs) such as docosahexaenoic and eicosapentaenoic acids (DHA, EPA) exert ischemic anti-arrhythmic effects. However, their mechanism of action remains unknown. The present study was designed to investigate their potential effect on the regulation of the late sodium current as the basis for their ischemic anti-arrhythmic activity. Human isoforms of wild-type SCN5A and ΔKPQ-mutated cardiac sodium channels were stably transfected in HEK 293 cells and, the resulting currents were recorded using the patch clamp technique in whole cell configuration. In addition to their effect to inhibit peak I_{Na}, acute application of DHA and EPA blocked veratridine-induced late sodium current (late $I_{Na\text{-}Verat}$) in a concentration – dependent manner with IC_{50} values of 2.1 ± 0.5 μM and 5.2 ± 0.8 μM, for DHA and EPA, respectively. Channels availability was reduced, resulting in a significant leftward shift of the steady-state inactivation curve by -10.0 ± 2.1 mV and -8.5 ± 0.2 mV for DHA and EPA, respectively. Similar inhibitory effects of DHA and EPA were also observed on late $I_{Na\text{-}KPQ}$. In addition to their role as blocking agents of peak I_{Na}, DHA and EPA reduced human late I_{Na}. These results could explain the anti-arrhythmic properties of DHA and EPA during ischemia or following ischemia-reperfusion.

Key words Na^+ channels – fatty acids – ischemia – long QT syndrome – persistent sodium current

Introduction

The sodium channel undergoes conformational changes in response to changes of membrane potential and of time [3]. Following a depolarization, from a resting closed state, channel opens instantaneously allowing Na^+ to flow following its electrochemical gradient. This conducting state of Na^+ channel is followed by a rapid nonconducting, absorbing, inactivated state. The channel then needs to recover from the inactivated state to be activatable again upon repolarization of the membrane potential. In addition, it has been shown recently that sodium current does not completely return to zero and hence a small sodium current (representing 1–2% of peak rapid current) flows and persists as depolarization is maintained [20, 27, 30–32]. This latter current is called persistent or late sodium current and is different from the sodium window current which is due to the overlap of the activation and inactivation curves of the fast sodium current. Thus, the sodium current is important not only in the triggering of the APs of ventricular myocytes but also to a smaller extent in the regulation of AP duration by maintaining the duration of the plateau phase and by contributing to the repolarization phase [1].

The late sodium current is also observed and increased under pathological conditions such as cardiac

BRC 676

hypoxia/ischemia. It has been demonstrated that ischemic metabolites such as lysophosphatidylcholine and palmitoyl-L-carnitine increase late sodium current in rat ventricular myocytes [16, 19, 31, 35, 36]. In addition to its role on action potential and arrhythmias, this increase in late I_{Na} results in an elevated $[Na^+]_i$ [12, 15, 39]. This, in turn, activates the Na^+/Ca^{2+} exchanger in its reverse-mode promoting Ca^{2+} overload, a well-described feature of ischemic/hypoxic cardiac cells. This increase in $[Ca^{2+}]_i$ is responsible for cell contracture and activation of signaling pathways leading to cell death. Therefore blockade of late I_{Na} may have powerful beneficial effects in reducing ischemic arrhythmias due to $[Ca^{2+}]_i$ overload [13]. Thus, changes of I_{Na} properties have profound repercussions on cardiac electrophysiology as observed during ischemia but also with inherited mutations of sodium channels such as in Brugada syndrome and long QT syndrome type 3 (LQT3). These syndromes are associated with abnormal repolarization and a high risk of ventricular arrhythmias and sudden death [1]. LQT3 syndrome is associated with the deletion of three amino acids in the intracellular linker between domains 3 and 4 (ΔKPQ) located closely to the IFM motif involved in the fast inactivation process [4, 11, 41].

Fish oil n-3 polyunsaturated fatty acids (PUFAs) are known to exert beneficial effects on arrhythmias [28]. The risk reductions in sudden death by PUFAs have been demonstrated by several randomized controlled trials. GISSI study, a randomized clinical trial investigated the effects of PUFAs supplementation in the secondary prevention of myocardial infarction. Treatment with PUFAs lowered mortality, and sudden cardiac death was reduced by 45 %. Contrary to beneficial effects of fish oil, DART-2 study reported that fish oil supplementation resulted in an increase in cardiac death in patients with angina pectoris [7]. Moreover, in subjects with implanted cardioverter defibrillators (ICD), fish oil supplementation resulted in a pro-arrhythmic response [29]. Two other trials reported that fish oil supplementation in patients with ICDs did not result either in a pro- or an anti-arrhythmic response [6, 21]. Finally, the reduction in cardiovascular mortality by PUFAs may be limited to reduction of sudden cardiac death in post-myocardial infarction. In addition, an experimental study demonstrated that PUFAs could specifically prevent ischemia-induced ventricular fibrillation in dogs [5, 22]. Recently, an inverse relationship between plasma PUFAs content and the risk of ventricular fibrillation in patients with ischemic heart disease and implantable cardioverter defibrillators, suggesting a role of PUFAs in preventing ventricular arrhythmias has been reported [8]. The stabilizing effect of PUFAs on cardiomyocytes excitability is due to a combined effect on several ion channels and transporters, but mainly on sodium and calcium channels [43, 45, 46]. These agents slightly hyperpolarize the resting membrane potential and increase the activation threshold of sodium channels in a way that the magnitude of the stimulus to trigger the action potential is increased. Moreover, in in vivo studies PUFAs have been shown to exert protective effect against "ischemic arrhythmias" [5]. Because of the roles of late sodium current in ischemic damage and in the genesis of arrhythmias are well established [16, 19, 31, 35], it appeared interesting to evaluate the effects of PUFAs, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) on the peak and late components of sodium currents. Sodium currents were studied in HEK 293 cells stably transfected either with human (h)Nav1.5 or with the mutant $hNa_v1.5$-ΔKPQ of the α-subunit channel. EPA and DHA significantly reduced peak I_{Na} as well as late sodium currents induced by veratridine or by the constitutive active $hNa_v1.5$-ΔKPQ channels.

Materials and methods

Animals used in this study were housed and tested in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility in strict compliance with all applicable regulations and the protocol was carried out in compliance with French regulations and with local Ethical Committee guidelines for animal research.

Cell culture and isolation of cardiac myocytes

HEK 293 cells stably transfected with human SCN5A (hH1A) were kindly provided by Pr. Abriel (University of Lausanne, CH). Stably transfected HEK 293 cells expressing $hNa_v1.5$-ΔKPQ mutant were obtained as described below. The HEK 293 cell line (ATCC CRL-1573) was permanently transfected with a receptor gene using a gene pulser transfection apparatus (Bio-Rad). Isolation of cardiac myocytes was performed as previously described. Cells were grown under standard conditions in Falcon plates. Cells were removed with trypsin-EDTA (Invitrogen, 0.125 % trypsin) in PBS. Cell suspension was diluted in culture medium containing: DMEM (Gibco), 10 % of heat-inactivated fetal calf serum (Gibco), 0.1 M L-glutamine (Serva), 0.04 M pyruvate (Sigma), 8.6 mM penicillin (Serva), 7 mM streptomycin (Serva) and 2 mM zeocine (Invitrogen). Cells were seeded in 35 mm Petri dishes at the density of 10000 cells per dish and cultured for 2 or 3 days.

Enzymatic isolation of cardiac myocytes was derived from [23]. Briefly, male Sprague-Dawley rats (220–250 g) were killed with an overdose of pentobarbital sodium (250 mg/kg i.p.), and the heart was rapidly excised and washed in ice-cold, oxygenated, Tyrode solution (in mM: NaCl 120, KCl 5, $MgCl_2$ 1, HEPES 20, Na-pyruvate 4.5,

glucose 20, pH 7.3) for 1 min. Hearts were then cannulated and successively perfused (at 37 °C) with the following oxygenated solutions: for 4 min with Tyrode solution, for 6 min with a nominally calcium-free Tyrode and for about 15 min with the same solution complemented with 1 mg/ml collagenase (Type IA, 1 mg/ml, Sigma Chemicals, St Louis, MO, USA) and protease (type XIV, 0.3 mg/ml, Sigma Chemicals, St Louis, MO, USA). When the heart became flaccid it was then washed with a 0.05 mM calcium-Tyrode solution for 5 min. Ventricles were then removed and cut into small pieces and kept at room temperature in KB solution (in mM: KCl 70, K glutamate 5, KH_2PO_4 20, $MgSO_4$ 5, $CaCl_2$ 0.08, EGTA 5, creatine 5, Na_2ATP 5, taurine 20, HEPES 10, D-glucose 10, pH 7.2 with KOH).

Patch clamp experiments

Voltage clamp

Sodium current was recorded using the patch clamp technique in the whole cell configuration. Micropipettes with resistance of 1–3 MΩ (when filled with the pipette solution, see composition below) were made from borosilicate glass capillaries (model GC 150 F, Harvard Apparatus LTD, UK). A patch clamp amplifier (Axopatch 200A, Axon instruments, Foster City, CA, USA) was used. The resistance in series with the cell membrane was not compensated. Neither capacitive nor leakage currents were compensated. Cell currents were digitized at 6 kHz and analyzed by computer (Desk-Pro 486/33 MHz, Compaq, Houston, TX, USA) with interactive software (ACQUIS 1, G. Sadoc, Paris, France).

For I_{Na} measurements in SCN5A-transfected HEK 93 cells, K^+ currents were abolished by a nominally K^+-free medium and by addition of CsCl. The internal solution (pipette) contained (in mM): NaCl 10, CsCl 110, $CaCl_2$ 1, HEPES 10, EGTA 10, Mg-ATP 5, D(+)-glucose 10, pH 7.3 (CsOH). The external solution used to superfuse the cells had the following composition (in mM): NaCl 30, CsCl 100, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10, D(+)-glucose 5, pH 7.4 (CsOH). For the recording of $I_{Na\text{-}KPQ}$, the electrochemical gradient for Na^+ was increased by raising the external concentration of Na^+ from 30 mM to 60 mM. For I_{Na} measurements in isolated rat ventricular myocytes, the internal solution contained (in mM): NaCl 5, $CaCl_2$ 1, CsCl 130, $MgCl_2$ 2, HEPES 10, EGTA 15, Mg-ATP 4, pH 7.2 (CsOH). The external solution contained (in mM): NaCl 25, CsCl 110, $CoCl_2$ 2.5, $CaCl_2$ 0.5, $MgCl_2$ 2.5, HEPES 10, 4 aminopyridine 5, D-glucose 10, pH 7.4 (CsOH).

To obtain rapid and uniform control of the membrane potential and to minimize voltage errors, I_{Na} was recorded using an external solution containing 30 mM NaCl. Moreover, small cells were selected and cells with I_{Na} amplitude > 10 nA were rejected. Because of the fast kinetic of activation of Na^+ channels, all experiments were carried out at room temperature (19–22 °C).

Sodium current was elicited by square depolarizing pulses of 350 ms duration from a holding potential of −110 mV to −30 mV delivered at a frequency of 0.2 Hz. In order to verify the stability of voltage-clamp, every five pulses, the holding potential was shifted to −90 mV for one pulse. For the study of whole cell current parameters, sodium currents were generated using a double-pulse protocol to obtain current-voltage (I-V) curves and steady-state inactivation and activation curves. Current density was calculated by dividing whole-cell current amplitude by whole-cell capacitance. From a holding potential of −110 mV, 350 ms depolarizing pulses to different membrane potentials (10 mV increments, i.e. the conditioning pulse, up to +40 mV) were followed by a 1 ms return to −110 mV and then by a 350 ms test pulse to −30 mV (potential where I_{Na} is fully activated). Data used for the I-V curves and activation curves were measured from the conditioning pulse. Activation curves were estimated according to the relation: $G_{Na} = I_{Na}/(V_m\text{-}V_{rev})$ where G_{Na} is the conductance, I_{Na} is the amplitude of the sodium current for the test potential V_m and V_{rev} is the apparent reverse potential for Na^+. Steady-state inactivation curves were plotted from data recorded during the test pulse. The data for activation and steady-state inactivation were fitted with a simple Boltzmann function: $I/Imax = \{1 + \exp[V_m\text{-}V_{0.5})/k]\}^{-1}$ where I/Imax is the relative current, $V_{0.5}$ is the half-maximum voltage of activation or inactivation and k is the slope factor. Wild-type rapid sodium current is designated as I_{Na}. Sodium current presenting an incomplete inactivation was induced either with the alkaloid veratridine ($I_{Na\text{-}Verat}$) or with ΔKPQ-mutation of $Na_v1.5$ channels ($I_{Na\text{-}KPQ}$). The following parameters of I_{Na} were measured: the peak I_{Na} and the late I_{Na}. The peak amplitudes were measured as maximal amplitudes during the first 5 ms of the depolarizing pulse, and the late I_{Na} as the mean current amplitude of the last 10 ms of the pulse (that is the magnitude of I_{Na} at 340 to 350 ms of the depolarizing pulse) and they were expressed as current density in pA/pF. It should be noticed that wild-type HEK 293 cells did not exhibit any peak and late inward sodium current (data not shown).

Drugs

Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and veratridine were purchased from Sigma. The vehicle used to prepare stock solution of DHA and EPA was ethanol (0.1 %), and DMSO was used for veratridine (0.1 %).

Statistical analysis

Statistical analysis was performed using SigmaStat and involved one-way analysis of variance using Tukey's method as post hoc test. The results are presented as mean ± SEM.

Results

Effects of DHA and EPA on peak sodium current

Voltage-activated I_{Na} with fast activation and complete inactivation were elicited in HEK 293 cells stably transfected with $hNa_v1.5$ coding for the α subunit of sodium channel. Fig. 1Aa illustrates two representative I_{Na} traces elicited by depolarizing pulses from a holding potential of –110 mV to –30 mV in the absence (vehicle) and presence of DHA (10 μM). Whole cell current activation and steady-state inactivation were determined using a double-pulse protocol. DHA at 10 μM reduced peak transient I_{Na} density at every potential in the range from –50 to + 10 mV as illustrated by the current-voltage (I-V) curves shown in Fig. 1Ab. The vehicle mean maximum I_{Na} density at –30 mV was –87.8 ± 12.0 pA/pF and was reduced by DHA (10 μM) to –17.4 ± 8.5 pA/pF (n = 6, $P < 0.05$). Voltage-activation parameters were affected by 10 μM DHA. As illustrated in Fig. 1Ac, the potentials of half-activation ($V_{0.5}$) were –40.2 ± 0.1 mV and –47.0 ± 0.1 mV (n = 3, $P < 0.05$) for vehicle and DHA (10 μM), respectively. DHA (10 μM) also caused a steady-state inactivation curve shift toward hyperpolarized po-

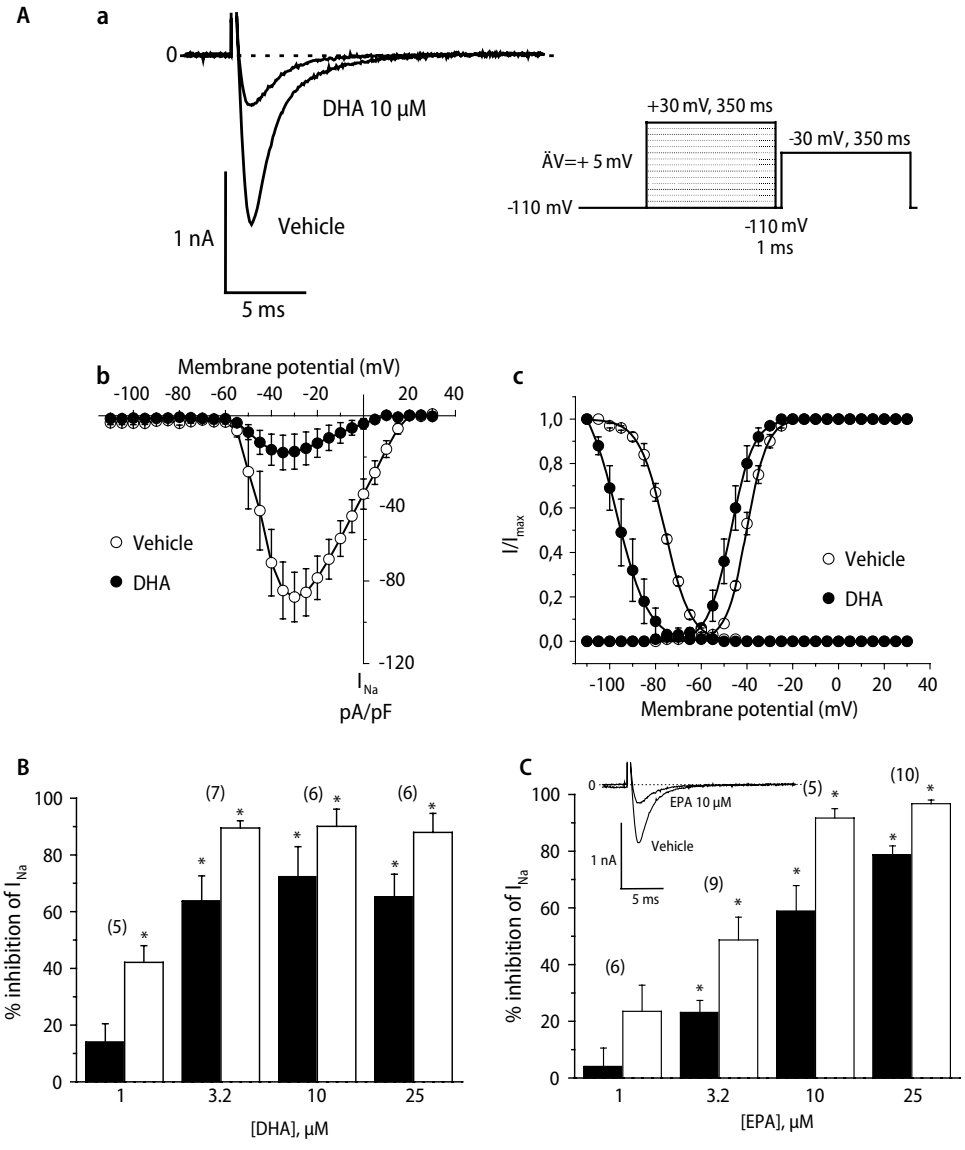

Fig. 1 Effects on DHA and EPA on peak I_{Na}. A, Effect of 10 μM DHA on peak I_{Na}. **Aa**, examples of transient sodium current recordings elicited by a depolarizing pulse from a holding potential of –110 mV to –30 mV in absence or in presence of 10 μM DHA. Whole cell current I–V (**Ab**) and activation and steady-state inactivation (**Ac**) curves were determined using a double-pulse protocol (**Aa**) before and after application of 10 μM DHA (n = 6). Data for activation and steady-state inactivation relationships were fitted to the Boltzmann equation as explained in the methods section. **B**, Bar graph summarizing the inhibitory effects of various concentrations of DHA on peak I_{Na} elicited from –110 (solid bars) and –90 mV (open bars). **C**, Bar graph summarizing the inhibitory effects of various concentrations of EPA on peak I_{Na} elicited from –110 (solid bars) and –90 mV (open bars). Examples of transient sodium current recordings elicited by a depolarizing pulse from a holding potential of –110 mV to –30 mV in absence or in presence of 10 μM EPA are shown in the inset. *, for $P < 0.05$. Number of experiments are indicated in parentheses.

tentials (Fig. 1Ac). The potentials of half-inactivation ($V_{0.5}$) were −75.9 ± 0.7 mV and −94.6 ± 0.2 mV (n = 3, P < 0.05) for vehicle and DHA at 10 μM, respectively. It should be pointed out that relatively few cells (n = 3) were used to study the steady-state availability (by contrast to n = 6 for I-V curve) because DHA at 10 μM was a potent inhibitor of I_{Na}, and hence only 3 of 6 cells were suitable for analysis. Inhibition of peak transient I_{Na} by DHA was concentration-dependent (Fig. 1B). Peak I_{Na} was reduced by 13.9 ± 6.6 % (DHA at 1 μM, n = 5, NS), by 63.7 ± 9 % (DHA at 3.2 μM, n = 7, P < 0.05), by 72.3 ± 10.6 % (DHA at 10 μM, n = 6, P < 0.05) and by 65.1 ± 8.1 % (DHA at 25 μM, n = 6, P < 0.05). Furthermore, consistent with the effects of DHA on steady-state availability of sodium channels, the inhibitory effect of DHA was more pronounced when the resting membrane potential was depolarized. For example at a holding potential of −90 mV the mean inhibition values of peak I_{Na} by DHA were 42.1 ± 5.9 % (n = 5, P < 0.05), 89.5 ± 2.6 % (n = 7, P < 0.05), 90.1 ± 6.1 % (n = 6, P < 0.05), 88.0 ± 6.7 % (n = 6, P < 0.05) at concentrations of DHA of 1, 3.2, 10 and 25 μM, respectively (Fig. 1B).

Similar results to those observed for DHA on peak transient I_{Na} were obtained with EPA. Fig. 1C (inset) illustrates representative I_{Na} traces elicited at −30 mV from a holding potential of −110 mV in absence and in presence of EPA at 10 μM. EPA reduced peak I_{Na} markedly. Peak I_{Na} was inhibited by 10 μM EPA at every potential in the range from −40 to + 20 mV with a mean maximum I_{Na} density at −30 mV of −160.2 ± 19.3 pA/pF and −65.2 ± 12.7 pA/pF (n = 5, P < 0.05, data not shown) for vehicle and EPA, respectively. Steady-state availability curves show that steady-state inactivation as well as activation curves were affected by 10 μM EPA, with a more pronounced effect on steady-state inactivation. Activation $V_{0.5}$ were −45.9 ± 0.3 mV and −50.2 ± 0.1 mV (n = 5, P < 0.05) for vehicle and EPA at 10 μM, respectively. Steady-state inactivation $V_{0.5}$ were −84.1 ± 1.5 mV and −100.2 ± 0.3 mV (n = 5, P < 0.05) for vehicle and EPA (10 μM), respectively. As summarized in the bar graph of Fig. 1C, EPA significantly reduced peak I_{Na} in a concentration-dependent. Peak I_{Na} (elicited at −30 mV from holding potential of −110 mV) was reduced by 4.0 ± 6.5 % (EPA at 1 μM, n = 6, NS), by 23.0 ± 4.3 % (EPA at 3.2 μM, n = 9, P < 0.05), by 58.8 ± 9.1 % (EPA at 10 μM, n = 5, P < 0.05) and by 78.7 ± 3.2 % (EPA at 25 μM, n = 10, P < 0.05) yielding an IC_{50} of 6.3 μM with an 95 % confidence interval of [0.31; 3.87 μM]. EPA was slightly more potent when I_{Na} was elicited from a holding potential of −90 mV, with an IC_{50} of 2.8 μM (95 % confidence interval of [0.43; 1.24 μM]).

Effects of DHA and EPA on persistent sodium current

Late sodium current was studied using two approaches. First, a pharmacological approach which consisted of inducing a late I_{Na} with the alkaloid veratridine ($I_{Na\text{-}Verat}$), and a second approach which consisted of using cells expressing the ΔKPQ mutation of Nav1.5 channels, a mutation known to cause gain of function resulting in an incomplete inactivation leading to appearance of late I_{Na} ($I_{Na\text{-}KPQ}$).

Veratridine-induced persistent sodium current: $I_{Na\text{-}verat}$

Fig. 2 shows typical traces of persistent sodium currents induced with veratridine (40 μM) and, the effects of DHA at 10 μM. After a stabilization period of 5 to 10 min, veratridine (40 μM) was applied to the bath solution. Application of veratridine induced a decrease of peak I_{Na} amplitude (Fig. 2Ba), an effect associated with a decrease of the current inactivation process (Fig. 2A). Veratridine binds to the open state of the Na^+ channels and increases open probability [28] leading to delayed inactivation and the induction of a late sodium current ($I_{Na\text{-}verat}$). As shown in Fig. 2B, application of DHA at 10 μM strongly decreased peak $I_{Na\text{-}Verat}$ amplitude as well as late $I_{Na\text{-}Verat}$. Peak $I_{Na\text{-}Verat}$ was decreased by DHA at 10 μM at every potential in the range from −50 to −5 mV. The mean peak $I_{Na\text{-}Verat}$ densities at −30 mV were −96.7 ± 26.7 pA/pF and −25.1 ± 6.0 pA/pF for vehicle and DHA at 10 μM, respectively (Fig. 3A). The late $I_{Na\text{-}Verat}$ was also reduced by DHA. The mean late $I_{Na\text{-}Verat}$ densities at −30 mV were −17.2 ± 4.0 pA/pF and −3.5 ± 1.1 pA/pF (n = 6, P < 0.05) for vehicle and DHA at 10 μM, respectively. Steady-state availability curves (Fig. 3B) show that activation of peak $I_{Na\text{-}Verat}$ was not affected by DHA at 10 μM. The mean values of $V_{0.5}$ were −48.7 ± 2.2 mV in the control and −49.9 ± 1.8 mV in the presence of DHA (n = 6, NS). By contrast, steady-state inactivation of peak $I_{Na\text{-}Verat}$ was shifted significantly to the left by DHA at 10 μM. The mean $V_{0.5}$ values were −82.5 ± 1.2 mV and −98.2 ± 1.5 mV (n = 5, P < 0.05) for vehicle and DHA, respectively. This modification of steady-state availability of channels led to a reduced Na window current (Fig 3B inset). Similarly, steady-state inactivation of late $I_{Na\text{-}Verat}$ was shifted significantly to the left by DHA at 10 μM with mean $V_{0.5}$ values of −83.3 ± 1.3 mV and −96.3 ± 1.0 mV (n = 5, P < 0.05) for vehicle and DHA, respectively (data not shown).

The effects of DHA on veratridine-induced late I_{Na} were concentration-dependent. Reduction of late $I_{Na\text{-}Verat}$ by DHA yielded IC_{50} value of 2.1 μM with 95 % confidence interval [ND, 3, 2 μM] (Fig. 3C). The potency of DHA to inhibit late I_{Na} was also observed in isolated ventricular cells from adult rat. In this context, DHA (10 μM) reduced veratridine-induced late I_{Na} by 29.2 ± 6.2 % (N = 6, P < 0.01) elicited at −30 mV from a holding potential of −90 mV (data not shown).

Similarly to what observed with DHA, application of EPA caused a concentration-dependent reduction of $I_{Na\text{-}Verat}$ which resulted in IC_{50} values of 5.2 μM with 95 %

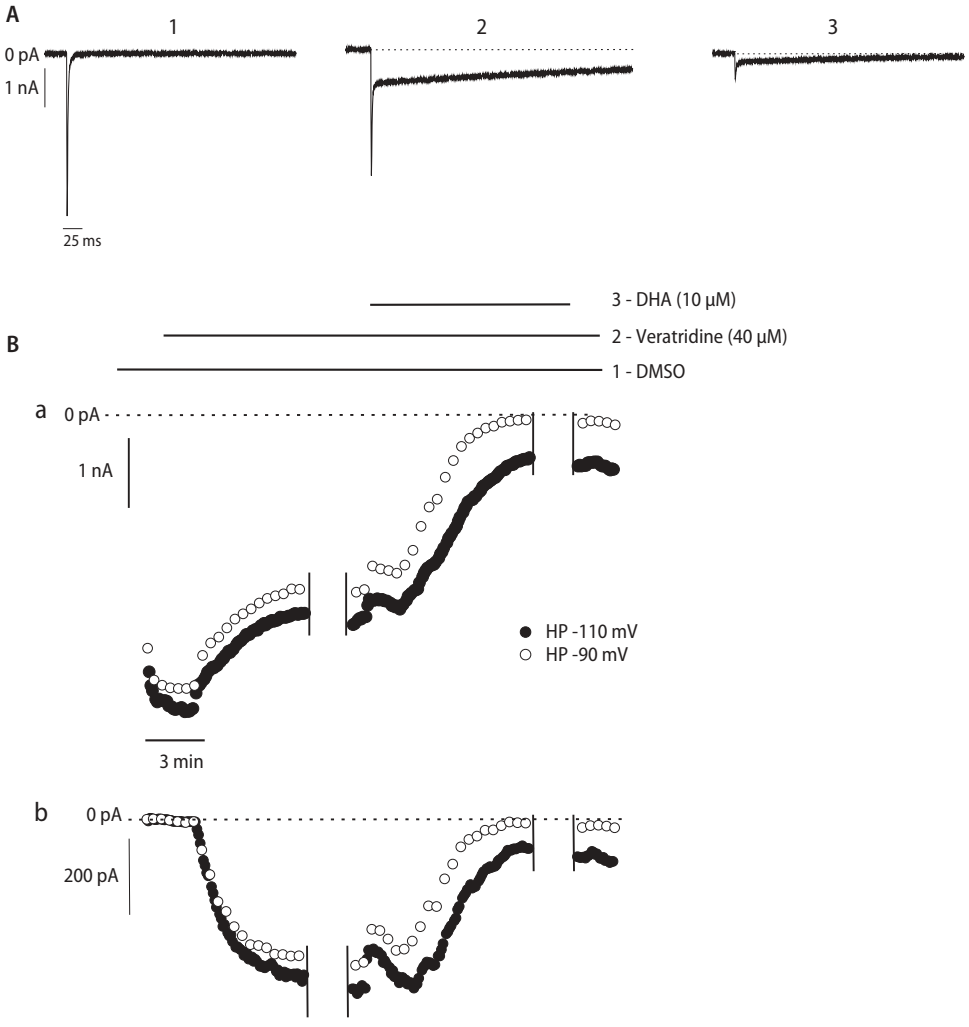

Fig. 2 Effects on DHA on late sodium current induced with veratridine. A, recordings of transient I_{Na} (**1**, DMSO 0.1%) and late I_{Na} [in absence (**2**, Veratridine 40 µM) or in presence of 10 µM DHA (**3**)] elicited by depolarising pulse from a holding potential of –110 mV to –30 mV corresponding to the recordings shown in B. **B**, examples of experimental recordings. Sodium current was elicited by depolarizing pulse to –30 mV from a holding potential of –110 mV every 5 sec (black circle) and from –90 mV every 25 sec (white circle) in order to follow the stability of voltage-clamp. After a stabilization period in presence of DMSO, veratridine was applied leading to a decrease in peak I_{Na} (**a**), and appearance of late sodium current $I_{Na\text{-}Verat}$ (**b**). Addition of 10 µM DHA decreased peak as well as late I_{Na} induced with veratridine.

confidence interval [3.4; 7.8 µM] (Fig. 4). The peak of $I_{Na\text{-}Verat}$ was decreased by EPA at 10 µM at every potential in the range from –50 to –5 mV. The mean peak $I_{Nap\text{-}Verat}$ densities –30 mV were –109.8 ± 38.2 pA/pF and –48.9 ± 22.4 pA/pF (n = 6, P < 0.05) for vehicle and EPA at 10 µM, respectively. The late $I_{Na\text{-}Verat}$ density was also reduced by EPA. The mean late $I_{Na\text{-}Verat}$ densities at –30 mV were –12.7 ± 1.7 pA/pF and –5.2 ± 3.2 (n = 6, P < 0.05) for vehicle and EPA at 10 µM, respectively. Steady-state availability curves (data not shown) show that activation of peak $I_{Na\text{-}Verat}$ was not affected by EPA at 10 µM. The mean values of $V_{0.5}$ were –46.5 ± 2.2 mV in control and –49.1 ± 2.3 mV in presence of EPA (n = 6, NS). By contrast, steady-state inactivation of peak $I_{Na\text{-}Verat}$ was shifted significantly to the left by EPA at 10 µM with mean $V_{0.5}$ values of –83.7 ± 0.9 mV and –95.7 ± 1.7 mV (n = 6, P < 0.05) for vehicle and EPA, respectively. Similarly, steady-state inactivation of late $I_{Na\text{-}Verat}$ was shifted significantly to the left by EPA at 10 µM with mean $V_{0.5}$ values of –82.7 ± 0.6 mV and –91.2 ± 1.9 mV (n = 4, P < 0.05) for vehicle and EPA, respectively.

ΔKPQ-induced persistent sodium current: $I_{Na\text{-}KPQ}$

$I_{Na\text{-}KPQ}$ with fast activation and incomplete inactivation was elicited in HEK 293 cells stably transfected with $hNa_v1.5$-ΔKPQ. In order to observe and to record persistent current, electrochemical gradient for Na^+ was increased by using 60 mM external Na^+ (instead of 30 mM). Under these conditions, it is well known that it is impossible to perfectly control the sodium current clamp. Thus, I-V curves, as well as voltage-activation and steady-state inactivation curves were not studied for the peak $I_{Na\text{-}KPQ}$. Fig. 5A illustrates representative $I_{Na\text{-}KPQ}$ traces elicited by a depolarizing pulse to –30 mV from a holding potential of –110 mV showing the incomplete inactivation of $I_{Na\text{-}KPQ}$ and the blocking effect of DHA at 10 µM.

Fig. 5B shows experimental recordings of $I_{Na\text{-}KPQ}$ before and after the application of DHA at 1 and 10 µM

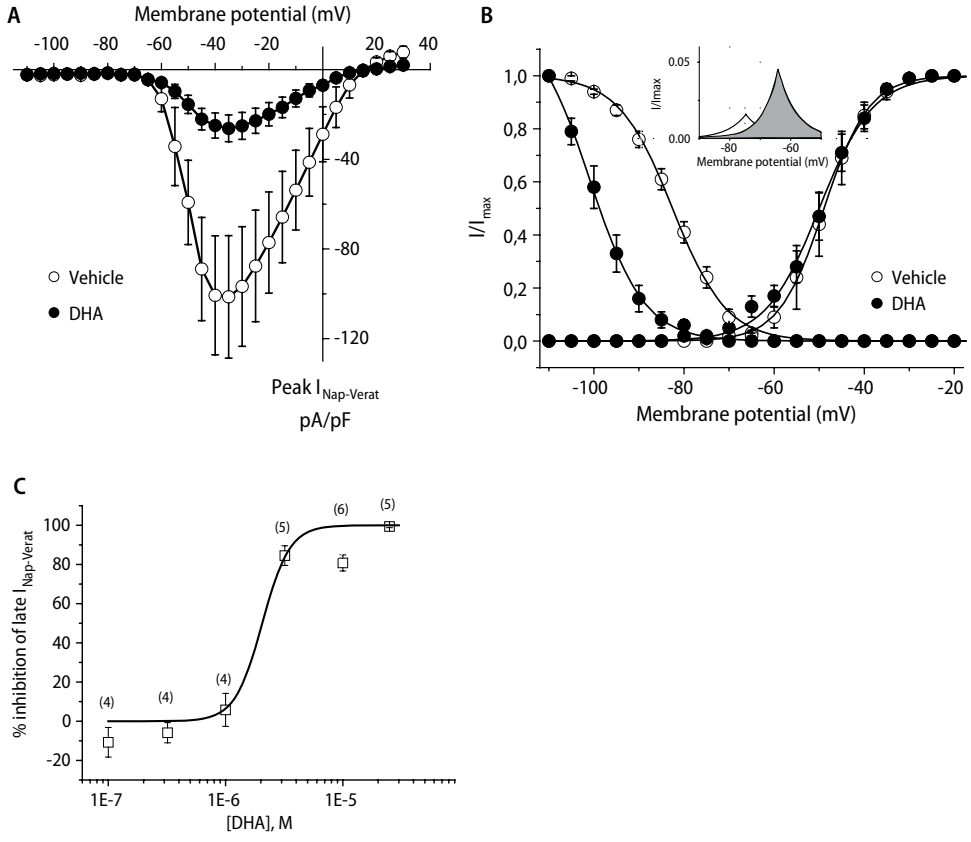

Fig. 3 Effects on DHA on late sodium current induced with veratridine. Peak $I_{Na\text{-}Verat}$-V (**A**) and activation and steady-state inactivation (**B**) curves were examined with a double-pulse protocol described in the methods section before and after application of 10 μM DHA. Data for activation and steady-state inactivation relationships were fitted to the Boltzmann equation as explained in the methods section. DHA shifted the steady state inactivation curves to hyperpolarized potentials leading to a smaller sodium window current as shown in the inset on expanded scale. Sodium window current in absence (control) is represented in gray and in presence of 10 μM DHA is represented in white. **C**, concentration-response curves of effects of DHA on late $I_{Na\text{-}Verat}$. Number of experiments are indicated in parentheses.

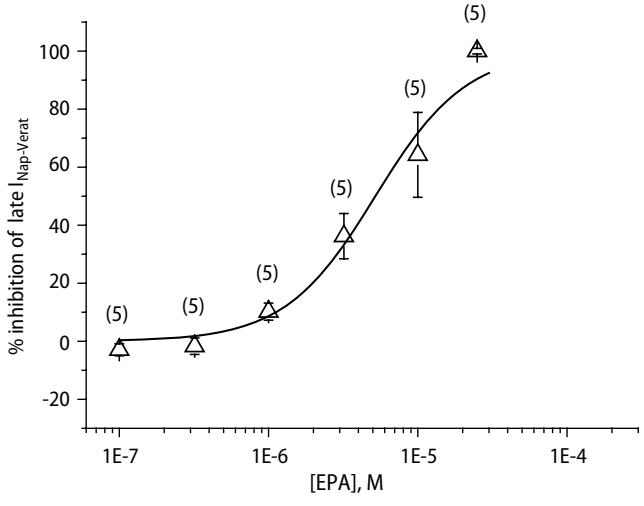

Fig. 4 Effects on EPA on late sodium current induced with veratridine. Concentration-response curves of the effects of EPA on late $I_{Na\text{-}Verat}$. Number of experiments are indicated in parentheses.

successively. Application of 1 μM DHA to the bath solution did not significantly modify late $I_{Na\text{-}KPQ}$ amplitude (Fig. 5B), whereas 10 μM DHA almost completely abolished late $I_{Na\text{-}KPQ}$ (Fig. 5A). Similar to DHA, EPA also reduced $I_{Na\text{-}KPQ}$ in a concentration-dependent manner (Fig. 6) but when compared to DHA, EPA was found to be much less potent than DHA in inhibiting late $I_{Na\text{-}KPQ}$.

Concentration-responses curves obtained for DHA on late $I_{Na\text{-}KPQ}$ (Fig. 6B) yielded an IC_{50} of 2.5 μM with a 95 % confidence interval of [1.1–5.1 μM] with a mean maximal inhibitory effect of 95.4 ± 3.25 %. A mean maximal inhibitory effect of 55.1 ± 7.14 was obtained with EPA on late $I_{Na\text{-}KPQ}$; hence IC_{50} value could not be determined.

Discussion

The main findings of this study are that DHA and EPA concentration-dependently reduced peak and late I_{Na} probably through interaction with the inactivated state of the sodium channels. Because ischemic cardiomyocytes are partially depolarized, sodium channels are mainly in their inactivated state favoring blockade by PUFAs. Since late I_{Na} is observed mainly in ischemic cardiomyocytes, blockade of the specific late I_{Na} by EPA and DHA may constitute the protective mechanism of these fatty acids against ischemia-induced ventricular arrhythmias and renders these compounds highly ischemia selective. Such an ischemia-selectivity has

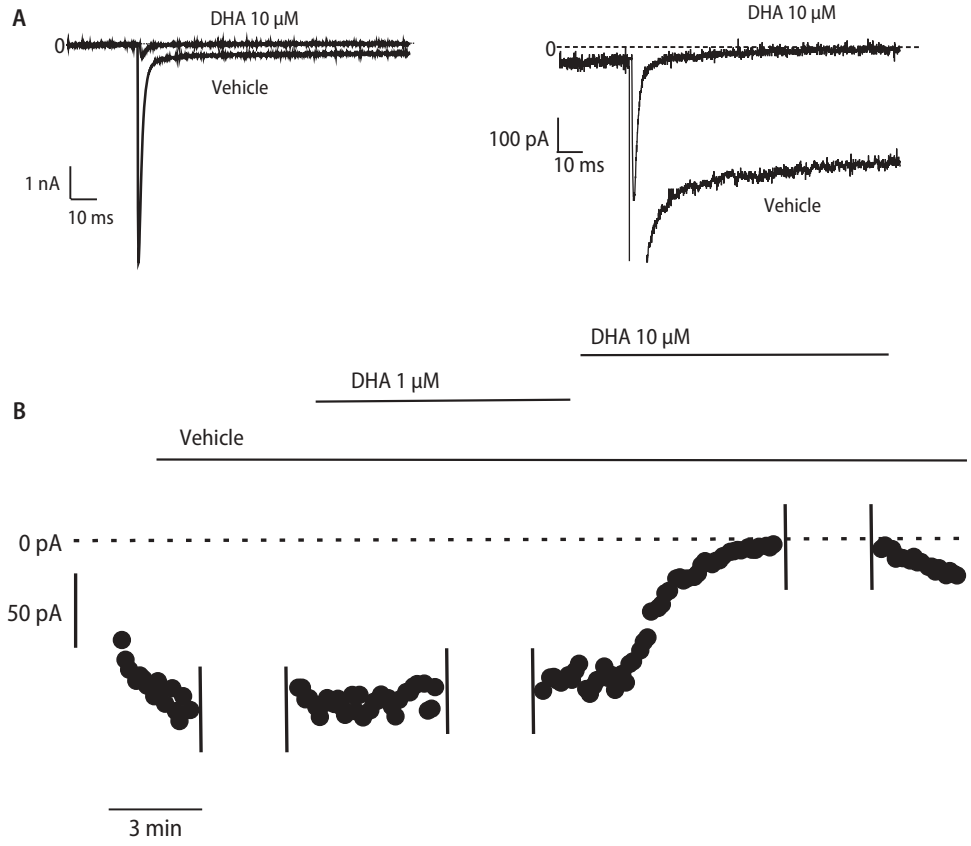

Fig. 5 Effects on DHA on late sodium current induced with ΔKPQ mutation. A, examples of sodium current, $I_{Na\text{-}KPQ}$, exhibiting an incomplete inactivation in absence or in presence of 10 µM DHA. DHA reduced peak $I_{Na\text{-}KPQ}$ as well as the late component of $I_{Na\text{-}KPQ}$ as illustrated on the expanded scale. **B**, examples of experimental recordings showing the effects of 1 and 10 µM DHA late $I^{Na\text{-}KPQ}$. Sodium current was elicited by a depolarizing pulse from a holding potential of −100 mV to −30 mV every 5 sec (black circle). After a stabilization period in presence of vehicle, application of 1 µM did not strongly reduce late $I_{Na\text{-}KPQ}$, whereas a higher concentration (10 µM DHA) completely inhibited late $I_{Na\text{-}KPQ}$.

been proposed already for the cardioprotection afforded by some calcium antagonists [17].

A single gene has been identified (SCN5A) which encodes the human cardiac sodium channel ($Na_v1.5$, [11]) that mediates both peak and persistent components of sodium current [20, 30]. A small fraction of I_{Na} persists throughout the plateau of the action potential and passes through a few Na^+ channels that fail to inactivate completely, and enter into either a long-opening or a bursting mode [25]. Despite its small amplitude, late I_{Na} appears to be crucial in controlling action potential duration and alterations of late I_{Na} have profound repercussions on cardiac electrophysiology.

First, our results confirm the effects of PUFAs on peak I_{Na} [22, 43, 45, 46], peak I_{Na} was reduced by acute application of DHA and EPA and this inhibition was concentration-dependent. Steady-state inactivation curve was shifted much more to the left than the activation curve leading to a reduced availability of channels, hence reducing the window I_{Na}. These results are in agreement with previous ones and confirm that DHA and EPA preferentially block the sodium channel in their inactivated states without or with minor effects on activation properties [46]. The mechanism of action of PUFAs on sodium channels is not yet elucidated but it has been shown that a single amino acid substitution in the D1-S6 region of the cardiac sodium channel affects the potency of fatty acid to block I_{Na} [43].

As peak I_{Na} is involved in the AP upstroke phase and propagation, it was believed that this effect of PUFAs on I_{Na} was the mechanism responsible for the beneficial effect of fish oil on cardiac arrhythmias [45]. Shift of the steady-state inactivation parameters stabilizes membrane excitability and renders the cell less prompt to initiate an ectopic AP. Although no clear picture of the mechanism of action of PUFAs has been established so far, a significant body of evidence, albeit not unanimous, pointed out that PUFAs can exert a protective effect on the heart, especially on ischemic diseases. The effects of DHA and EPA on steady-state inactivation curves were often used to explain the mechanism of action of n-3 fatty acids [43, 46]. However, this mechanism is not sufficient to discriminate between the effects of n-3 fatty acids from those of lidocaine and flecainide, compounds also known to interact with the inactivated state of Na^+ channel but are only weakly active against ischemia-induced ventricular arrhythmias. However, clinical studies have reported in humans that PUFAs decreased the risk of cardiac sudden death from ventricular arrhythmias [28] and that they could prevent ischemia-induced ventricular fibrillation in dogs [5, 22], which differs from the effects of lidocaine and flecainide, both peak I_{Na}

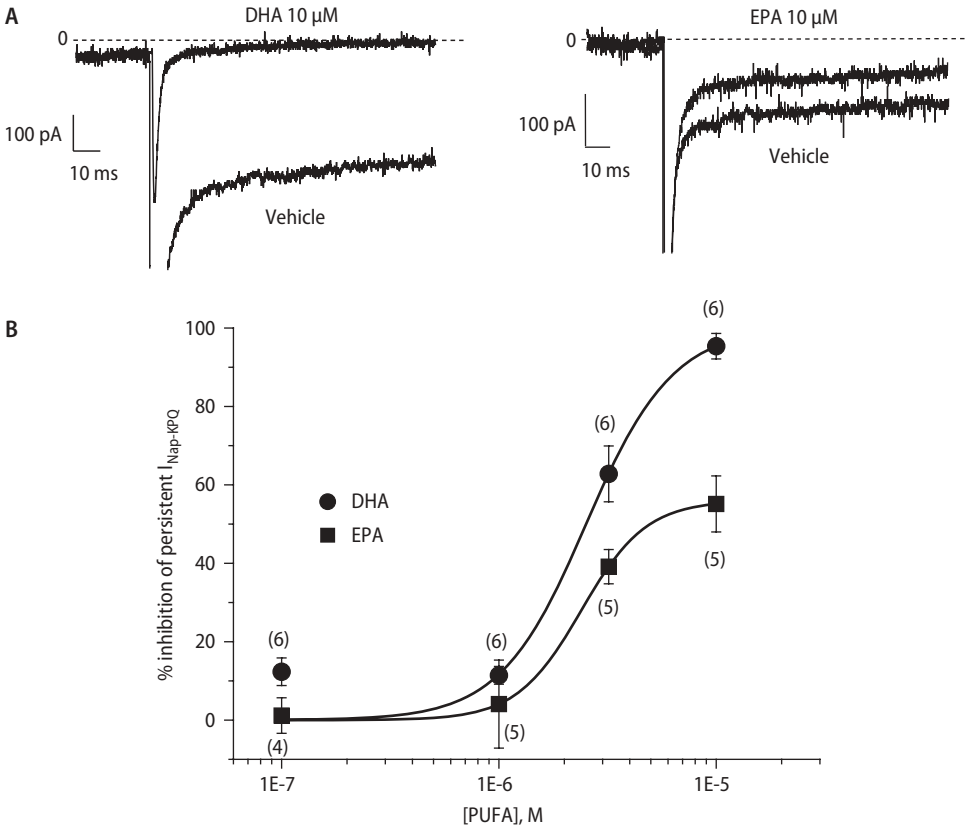

Fig. 6 Effects on DHA and EPA on late sodium current induced with ΔKPQ mutation. A, examples of I_{Na-KPQ} recordings in absence or presence of 10 µM DHA and 10 µM EPA and corresponding concentration-response curves on late I_{Na-KPQ} (**B**). Number of experiments are indicated in parentheses.

blockers. Moreover, in vivo studies have demonstrated a protective effect of PUFAs against "ischemic arrhythmias" in which late I_{Na} may be involved [5].

Second, beside the effects on peak I_{Na} the present results demonstrate that acute application of DHA and EPA concentration dependently reduced late $I_{Na-Verat}$ (mimicking its activation evoked by ischemia [15]) and by using the mutant $hNa_v1.5$-ΔKPQ. In addition, EPA and DHA significantly shifted the steady-state inactivation curve of late I_{Na} in a hyperpolarizing direction and maintained the rapid kinetics of inactivation for the residual peak current suggesting the elimination of the portion of non-inactivated currents. These findings show that DHA and EPA essentially abolish the late I_{Na} of the veratridine-activated current and of the (ΔKPQ) mutant and result in partial restoration of the inactivation process. In addition, DHA and EPA failed to affect the activation of I_{Na-KPQ} markedly. These results are in full agreement with those reported by Xiao et al. [44]. They characterized a mutant L409C/A410W of the α-subunit of human cardiac Na^+ channels which share a long lasting I_{Na}. EPA significantly shifted the steady-state inactivation of the mutant by –19 mV which is similar to the present results concerning $I_{Na-Verat}$. Collectively, these observations demonstrate that DHA and EPA concentration dependently inhibit late I_{Na} and stabilize $Na_v1.5$ channels in their inactivated state.

Late I_{Na} amplitude is increased in patients with an history of cardiac ischemia [31, 39] and in some patients with mutations in the SCN5A gene [4]. It is now well-established that the late I_{Na}-induced Na^+ loading is converted in Ca^{2+} overload through the activation of the reverse mode of the Na^+-Ca^{2+} exchanger [15, 39]. Therefore, enhancement of late I_{Na} is observed and is associated with arrhythmogenesis and left ventricular dysfunction in a variety of pathological states, including heart failure [37, 44], following hypoxia [16, 31, 35], after exposure to free oxygen radicals [31], amphiphiles such as lysophosphatidyl-choline [19] and palmitoyl-L-carnitine [39], and in ischemia remodeled myocytes [38]. Likewise as cellular damage caused by ischemia arises, action potential abnormalities such as early and delayed afterdepolarization are commonly observed. Computer models suggest that increases in late I_{Na} would cause significant lengthening of the action potential duration [32] possibly leading to afterdepolarizations. Indeed, it was shown that early afterdepolarizations are depressed by a reduction in external Na^+ concentration, by TTX [18] or by ranolazine [2, 9] both late I_{Na} blockers confirming the involvement of such a current. In addition, veratridine or ATXII, which causes persistent activation of sodium channels, induces early afterdepolarizations that can be completely eliminated with TTX [39] or ranolazine [33]. Interestingly, TTX also abolishes early af-

terdepolarizations in myocytes obtained from patients with heart failure [27]. Taken together, these observations have established that late sodium current is of first importance in ischemic arrhythmogenesis. An increase in late I_{Na} is also associated with congenital diseases (e.g. LQT3 syndrome) owing to mutations of the cardiac sodium channel gene SCN5A [41]. LQT3 syndrome is associated with the deletion of three amino acids in the intracellular linker between domains 3 and 4 (ΔKPQ) located closely to the IFM motif involved in the fast inactivation process [4, 36, 41]. Thus, ΔKPQ mutations induced an activation of late I_{Na} which is associated with abnormal repolarization and a high risk of ventricular arrhythmias and sudden death. Through blockade of late I_{Na} and restoration of a normal inactivation processing, DHA and EPA may specifically exert potent antiarrhythmic properties in ischemic depolarized cardiomyocytes. In these cells, the resting membrane potential is close to the threshold for the gating of the sodium channels so that any small depolarizing stimulus might initiate an action potential which may trigger fatal arrhythmias. In the presence of DHA and EPA, the shift of the steady state of inactivation of I_{Na} and late I_{Na} to physiologically unobtainable hyperpolarized potentials can eliminate abnormal stimulus and can prevent arrhythmias [43, 46]. In addition, by reducing the amplitude of late I_{Na}, DHA and EPA prevent the sodium loading which have been reported to be dramatically increased in mutant like ΔKPQ [26].

It should be noticed that potencies of DHA and EPA in inhibiting late I_{Na} differ on veratridine-induced late I_{Na} and on late $I_{Na\text{-}KPQ}$. Whereas DHA and EPA are almost equipotent in inhibiting veratridine-induced late I_{Na}, DHA but not EPA completely block late $I_{Na\text{-}KPQ}$. This may be due either to difference between the chemical structures of DHA and EPA or to the way of inducing persistent sodium current. One may suggest that conformation of veratridine-bound Na^+ channels is different from that of ΔKPQ-Na^+ channels and that the site of action of DHA and EPA may be exposed differently to PUFAs. As PUFAs are amphiphilic compounds they can act on plasmalemmal proteins in two ways depending on whether they are incorporated into the membrane or they are circulating in the extracellular medium. Most results attempting to elucidate mechanism involved in effects of PUFA on the heart were obtained from acute exposition of cells to PUFAs [45] and only relatively few results were from dietary exposition [10, 40]. It has been reported recently by Verkerk et al. [40] that a fish oil diet increases PUFAs content of ventricular myocytes and that is accompanied by a shortening of AP and alteration of several ion currents. Although Na^+ current amplitude was unaffected by a fish oil diet, the steady-state inactivation of I_{Na} was shifted to more negative potentials. These effects of PUFAs are independent of circulating PUFAs and additional effects may be observed with circulating PUFAs. Further investigations are needed to investigate these effects of circulating PUFAs and dietary exposure as performed by Verkerk et al. [40] as a function of ischemic state of the heart. However, one cannot excluded that both the acute exposition and the dietary exposure could lead to the same modulation of late I_{Na}.

In addition to this, some limitations of our study remain. First, in order to perfectly clamp the fast activating Na^+ current, experiments were performed at room temperature. We may suggest that at physiological temperature where kinetics of Na^+ channels are more rapid, and membrane fluidity more important, the incorporation of PUFAs would be greater. Thus in our experimental conditions, the effects of membrane incorporation of PUFAs would only be under-estimated. Second, it has been shown that fatty acid block of sodium channels may be altered or modified by the presence or absence of the auxiliary β_1-subunit [47]. The β_1-subunit modifies the kinetics of Na^+ channels in a way that activation and steady-state inactivation parameters are shifted towards depolarized potentials. It is now known that PUFAs (EPA and DHA) act on Na^+ channels by shifting the steady-state inactivation curve to hyperpolarized potentials. This may explain the less potent effect of PUFAs on $I_{Na\alpha+\beta}$ than on $I_{Na\alpha}$. This is particularly true with EPA, but not with DHA. The blockade effect of DHA on I_{Na} does not seem to depend on the presence of the β-subunit. Even if we may argue that our model does not mimic perfectly the physiological environment of human cardiomyocytes membranes, we can suggest that our results about DHA are expected to be more relevant than those of EPA which may be over-estimated. Experiments on isolated ventricular myocytes from adult rat confirmed the potency of DHA to reduce late I_{Na} in a more physiological environment. Moreover, differences in the potencies of DHA and EPA blockade of I_{Na} as a function of the presence or not of the β subunit may be due to their aptitude to displace the steady-state inactivation curve.

In conclusion, it has now become apparent that late component of I_{Na} plays an important role in the genesis of ischemia-induced ventricular arrhythmias. The present results demonstrate that late I_{Na} represents an attractive target to explain the protective effect of DHA and EPA reported in the clinical trials [28]. Therefore, such blockers of this current would be expected to provide protection from Na^+-induced Ca^{2+} overload and cell damage during ischemia and reperfusion. As a consequence, DHA and EPA may exert novel antiarrhythmic properties, particularly in the presence of ischemic depolarized cardiomyocytes.

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